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Quantum Dots Crack the Influenza Uncoating Puzzle

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One hundred years ago, the devastating 1918/19 Spanish influenza pandemic took the lives of 50-100 million people, or 3-5 % of the world population (1). Influenza A virus (IAV) pandemics occur when an animal IAV crosses the species barrier, usually by acquiring a new genetic trait by reassortment (2). According to the 2017 National Risk Register of Civil Emergencies of the United Kingdom, the predicted impact severity of a full-blown influenza pandemic is at the highest level; greater than that of coastal flooding (tsunami), major industrial accidents, and attacks on crowded places or transport. Has the advent of vaccines and antivirals truly increased our preparedness for the next influenza outbreak, while we still struggle to predict which seasonal strains will circulate in the next flu season? Needless to say, much is unknown about the cell biology of influenza infection and how the virus interacts with the multitude of host cellular processes that enable infection. Virus entry mechanics can be explored as a target for anti-influenza therapy. To complement virus-host interaction studies using cell biology, biochemistry, and structural biology, robust live imaging strategies that offer high temporal and spatial resolution are essential. However, the influenza RNA genome is intolerant to large genetic insertions, and efforts to rescue viruses using GFP fused to viral core proteins have had limited success (3). This has unfortunately delayed the growth of influenza entry live imaging studies in the field. In PNAS, Qin et al. (4) develop a nanotechnology that labels influenza viral ribonucleoproteins (vRNPs) with Quantum Dots (QDs) – an approach that will advance the mechanistic understanding of influenza virus entry using live fluorescence microscopy.

Influenza virus is a member of the *Orthomyxoviridae* family. It is an enveloped, single-stranded negative strand RNA virus with 8 pin-like genomic segments called vRNPs protected by a single M1 matrix shell. Each vRNP consists of oligomeric NP, viral RNA and viral polymerase made of PA, PB1, and PB2 subunits (5). A single NP molecule binds 24-27 nucleotides *in vivo* (6, 7) and also has two sets of nuclear localisation signals (NLSs), ensuring robust nuclear import of uncoated vRNPs during entry (8, 9). vRNPs are 10 nm in width and 30-110 nm in length (10), and package themselves into a revolving cylinder-like orientation adopting a typical “7 + 1” arrangement with 7 vRNPs surrounding a central one (11, 12). All 8 vRNA segments contain unique packaging sequences residing mainly in the 3' and 5' termini of each vRNA (13), ensuring packaging of 8 distinct vRNPs into budding particles. It is the swapping of these vRNP segments that contribute to genetic reassortment between strains that co-infect the same cell (2).

Influenza virus uncoating can be broken down into three steps: priming, M1 shell uncoating, and vRNP debundling (**Fig. 1, highlighted in cyan**). In the priming step, the decreasing pH during endocytic trafficking renders the viral core uncoating-competent (14, 15). Opening of the M2 ion channels in the viral envelope allows influx of protons and potassium ions into the viral core, altering the core so that M1 and the vRNPs can dissociate once the core is delivered to the cytosol by fusion (14, 16-19). Priming is critical for infection since if the M2 channel is blocked by amantadine, viral fusion still happens but the viral particle remains stuck in late endosomes (15). After HA activation and fusion, M1 shell disassembly and vRNP debundling take place on the cytosolic surface of late endosomes (20, 21). Following fusion, unanchored ubiquitin chains are exposed from the viral core and activate histone deacetylase 6 (HDAC6) and the aggresome processing machinery (20). HDAC6 links the M1 shell to actomyosin, dynein and the microtubule network, and shearing forces from molecular motors break apart the shell. Following M1 shell uncoating, transportin 1 (TNPO1), a member of the importin β family proteins (22, 23), removes residual M1 from vRNPs by binding a PY-NLS signal at the

M1 N-terminus (21). This debundles vRNPs and facilitates their nuclear import by importin α/β via the classical NLS pathway (8).

Previously, microinjection of dye-labeled vRNPs into the cytoplasm and fluorescence in situ hybridization (FISH) have been used to study the behaviour of incoming vRNPs (24, 25). FISH studies of incoming vRNPs suggested that the separate vRNAs travel together until they reach the nucleus (25). Super-resolution imaging of incoming particles showed that vRNPs debundle before reaching the nucleus (21). When purified influenza vRNPs free of M1 were microinjected into cells, their nuclear accumulation was observed after 1 h of injection (26). Cores that consisted of vRNPs and M1 could not be imported into the nucleus. Thus the removal of M1 causes the dissociation of vRNPs from each other, ensuring their passage through the nuclear pore (14, 26). Single-particle tracking of microinjected, fluorescently labelled vRNPs, showed that vRNPs undergo multiple rounds of binding and release before finally being translocated through the nuclear pore (24).

In PNAS, Qin et al. (4) tackle the vRNP debundling puzzle by generating infectious influenza virions that package vRNPs non-covalently labelled with a QD via the PA subunit of the polymerase. Their approach enables long-term imaging of individual vRNP segments in living cells from viral attachment to nuclear import. Live imaging single incoming vRNPs over an extended period of time requires dye brightness, photostability and a high signal-to-noise ratio, in all of which QDs are superior compared to GFP. How are QDs incorporated into infectious influenza virions? The IAV PR8 strain was genetically manipulated to express a biotin acceptor peptide (BAP) fused to the polymerase PA subunit. The rescued rPR8-PA-AP virus was genetically stable over multiple passages, and was thus used to replicate in BirA-expressing MDCK cells in the presence of biotin. Shortly after, streptavidin-coated QDs (SA-QDs) were introduced into these cells by lipofection to allow non-covalent binding of the QDs to biotinylated PA. Influenza virions generated using this method packaged up to five QDs in a single particle. Many (60%) virus particles, however, contained only one QD. Those that

contained two QDs of different colour were used to live image vRNP uncoating. Co-detection with Rab7-ECFP revealed that the two different segments that colocalised in endosomes separated into single entities as they left the Rab7 compartment. If combined with viral fusion detection, the dual QD virions will prove even more informative. It is likely that soon after fusion M1 shell breakage and vRNP debundling take place efficiently on the cytosolic surface of late endosomes by the action of host factors HDAC6 (20) and TNPO1 (21). After debundling, the vRNPs undergo a three-stage movement as they import into the nucleus.

In summary, this study is important because it provides continuous documentation of the vRNP's journey from late endosomal escape to nuclear entry (4). The approach, however, has limitations. The separated QD signals during uncoating can indicate two sub-bundles of 1+7, 2+6, 3+5, 4+4, or several sub-bundles e.g. 2+3+3, and not necessarily 8 single segments. Further, QDs are 2-10 nm in diameter and their virion incorporation decreases viral replication significantly. The physical presence of QDs will influence the properties of vRNP-vRNP interactions that regulate debundling mechanics. Thus a multifaceted approach using alternative labelling techniques such as FIAsh (27), wild-type viruses, and cellular loss-of-function experiments using siRNA and CRISPR/Cas warrants deeper investigation before we conclude the precise mechanism of influenza vRNP debundling. However, this study expands the toolbox that allows researchers to visualise virus-host interactions during virus entry at single molecule resolution in combination with host factors of mechanistic interest.

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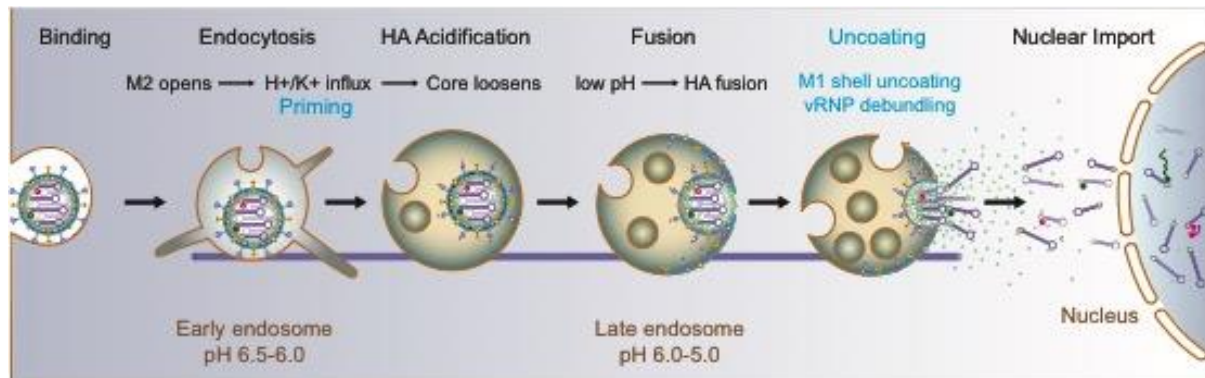


Figure Legend

Figure 1. Stepwise entry and uncoating of IAV. Influenza virus is an enveloped single-stranded negative strand RNA virus. It contains within its M1 matrix shell 8 genomic segments called vRNPs that are bundled together. When infecting a new cell, the IAV virion binds to sialic acids on cell surface receptors and triggers its own endocytosis into early endosomes via clathrin-mediated or macropinocytic uptake. Endosome maturation is key to successful virus entry and uncoating. The decreasing pH in endosomes opens the M2 ion channels on the viral membrane allowing influx of protons and potassium ions into the viral core, altering the core so that M1 and the vRNPs can dissociate once the core is delivered to the cytosol by HA-activated fusion. After fusion, cytosolic uncoating factors HDAC6 and TNPO1 promote further disassembly of the M1 shell and vRNPs, respectively. The real-time entry and uncoating of vRNPs can be tracked and dissected in detail, by imaging two individual vRNPs labelled with a QD (shown inside the particle as large green and red circles). Figure adapted from (28).